EXHIBIT E

Related Concept

- fungi: assessment, sampling and analysis
- fungi: MVOC - microbial volatile organic compound
- VOC: measurement and identification
- measurement: GC-MS
- measurement: SPME

Related References

- Lord, H. L., and Pawliszyn, J. (0). **Recent advances in solid phase microextraction and membrane extraction with a sorbent interface**
- Nilssona, T., Larsenb, T. O., Montanarellac, L. and Madsena, J. Ø., (1996), **Application of head-space solid-phase microextraction for the analysis of volatile metabolites emitted by Penicillium species**
- Pawliszyn, J., (2001). **Solid phase microextraction**

Related Articles

- PCR – polymerase chain reaction

Essay:

SPME - Solid Phase Microextraction

"This is a newly developed technique that is undergoing extensive research, it has been gaining in popularity over the last few years due to: Speed – Equilibration can be reached in only 2-30 minutes – ideal for quick screening. Sensitivity – Parts per trillion detection limits have been attained, with an ion-trap detector. The method which was invented in the early 1990's by Prof. Janusz Pawliszyn from the University of Waterloo in Ontario, Canada, uses a small segment of fused silica fibre coated with an appropriate material such as polydimethylsiloxane, this is then held in a syringe-like device. The extraction of analytes from various matrices is then undertaken and the introduction of these into a chromatographic system for analyses is the final step. No solvents are needed in this process, and analyte extraction and pre-concentration are combined in one single step. SPME fibers is a 1 or sometimes 2cm long fused silica fibre coated with a polymeric phase." -- http://www.chemsoc.org/exemplarchem/entries/2002/Garner/

"Solid-phase microextraction is a sample preparation and sample introduction method in which analytes partition from a sample into a polymer, coated on a fused silica rod of typically 1 cm length by 100 µm diameter. The fibre is fastened into the end of a fine stainless steel tube contained in a syringe-like device, and protected by an outer stainless steel needle. The device's plunger is depressed to expose the fibre to the sample matrix, retracted at the end of the sampling time, and then depressed again to expose the fibre to a desorption interface for analysis, typically by GC or HPLC."-- http://www.science.uwaterloo.ca/chemistry/pawliszyn/Research/MESI/Recentadvances.htm

Chen and Pawliszyn first introduced the technology of coupling standard SPME fibre sampling to HPLC analysis in 1995 6. (J. Chen and J. Pawliszyn, Anal. Chem. 67(15), 2530-2533 (1995)).

SPME is an extraction technology that combines sampling and sample preparation. Since it conception, SPME has been widely used for research applications in pharmaceutical, food, aroma, forensic, environmental and physicochemical properties. Three books on SPME summarize the theory, applications and many practical considerations (1. J. Pawliszyn , Solid Phase Microextraction- Theory and Practice. , Wiley-VCH, New York, USA (1997). 2. J. Pawliszyn (Editor), Applications of Solid Phase Microextraction, The Royal Society of Chemistry, Hertfordshire, UK, 1999. 3. S.A. Scheppers-Wiercinski (Editor), Solid Phase Microextraction: A Practical Guide, Marcel Dekke, New York, USA, 1999) -- Koziel and Novak, 2002, Sampling and sample-preparation strategies based on solid-phase microextraction for analysis of indoor air provided an excellent review

tidbits:

Research publications on SPME at PubMed

Web Links:

PubMed
"service of the National Library of Medicine, includes over 14 million citations for biomedical articles back to the 1950's. .. links to many sites providing full text articles and other related resources"

Solid Phase Microextraction

Brittany Hartzell

A new method in sample preparation should have the following analytical performance characteristics:

- Efficient
- Selective
- Applicable to various compounds and matrices
- Allow for simple automation and field analysis
- Easy to use
- Inexpensive
- Compatible with a wide range of analytical instruments
- Fast
- Use a minimal amount of solvent or be solvent-less
- Few steps

Introduction to SPME

Arthur and Pawliszyn developed this microscale technique in the late 1980's. They introduced it as a solvent-free sample preparation technique that could serve as an alternative to traditional extraction procedures such as liquid-liquid extraction, purge and trap, static headspace, and SPE procedures.

Basic principle: To use a small amount of the extracting phase, usually less than 1μL,while the sample volume can be very large. The extracting phase can be either high molecular weight polymeric liquid (similar to the sp in chromatography) or it can be solid sorbent (typically of high porosity to increase the surface area available for adsorption).

SPE vs. SPME

SPE (solid phase extraction) is a commonly used sorbent extraction technique.

- It is a total extraction technique- *all* of each analyte is transferred to solid extraction phase
- Analytes are extracted together with interfering compounds by passing an aqueous matrix through a plastic cartridge containing dispersed sorbent on a particulate support
- A selective organic solvent is used to remove interferences first, and then another solvent is chosen to wash out the target analytes
- Attractive features: simple, inexpensive, can be used in the field, can be automated, and uses relatively little solvent
- Limitations: low recovery- resulting from interaction between the sample matrix and analytes, some solvent is still necessary, and plugging of the cartridge by solid and oily components

On the other hand, SPME preserves all of the advantages of SPE while eliminating the main disadvantages of low analyte recovery, plugging, and solvent use.

Instrumentation

SPME consists of basically two main steps: 1) Equilibration of the analyte(s) between the fiber coating (extracting phase) and the sample matrix and 2) Desorption of the concentrated analyte(s) into an analytical instrument.

SPME steps in more detail:

1) The fragile fiber is initially withdrawn into the steel syringe needle
2) The sample septum is pierced, the coated fiber is extended into the sample solution for a set time, (typically 2-15 minutes for liquid samples) where the analytes are adsorbed by the fiber until an equilibrium is reached
   - Amount of analyte extracted by coating at equilibrium is determined by the magnitude of the partition coefficient of the analyte between the sample matrix and the coating material
3) The fiber is drawn back into the protective needle and the needle is withdrawn from the sample container
4) The needle is injected into the sample port of an analytical instrument, the fiber is extended, and the analytes are desorbed

SPME Theory

The principle behind SPME is the partitioning of analytes between the sample matrix and extraction medium.

To simplify the theory discussion we can assume the vial containing the sample is completely filled (no headspace is present). If a liquid polymer coating is used, we can use the following equation to relate the amount of analyte adsorbed by the coating at equilibrium to its concentration in the sample:

$$n = \frac{K_{fs} V_f C_o V_s}{K_{fs} V_f + V_s} \qquad \text{Eq. 1}$$

n: the mass of the analyte absorbed by the coating
V_f: volume of the coating
V_s: volume of the sample
K_{fs}: the distribution constant of the analyte between the coating and the sample matrix
C_o: the initial concentration of the analyte in the sample

As can be seen from this equation, there exists a linear relationship between the amount of analytes absorbed and their initial concentration in the sample.

Coatings used in SPME typically have strong affinities for organic compounds and therefore, have large K_{fs} values for targeted analytes. This means that SPME is selective and has a very high concentrating effect. However, many times the K_{fs} values are not large enough to exhaustively extract most analytes in the matrix and only through proper calibration can SPME be used to accurately determine concentrations of target analytes. Calibration can be by the external standard method in a relatively clean sample and by standard addition or internal standards in a more complex matrix.

If V_s is very large ($V_s >> K_{fs} V_f$):

$$n = K_{fs} V_f C_o \qquad \text{Eq. 2}$$

This means that when the volume of the sample is very large, the amount of analyte extracted by the fiber coating is not related to the sample volume . This feature, combined with its simple geometry makes SPME ideally suited for field sampling and analysis because the fiber can be exposed to air or dipped directly into a lake or river, without collecting a defined sample volume prior to analysis.

Non-Equilibrium Case:

SPME can still be used for quantitation in this case

$$n = [1 - \exp(-A \frac{2m_1m_2KV_f + 2m_1m_2V_s)}{m_1V_sV_f + 2m_2KV_sV_f}] \frac{KV_fV_s}{KV_f + V_s} C_o$$

A = surface area of polymer coating, m_1 = mass transfer coefficient in sample matrix, $m_1 = D_1/\delta_1$, m_2 = mass transfer coefficient in polymer coating, $m_2 = D_2/\delta_2$, D_1, D_2 = diffusion coefficients in sample matrix and polymer coating, δ_1, δ_2 = diffusion layers in sample matrix and polymer coating

This eqn. shows that n is proportional to C_o, if adsorption time and the agitation method is held constant for each sampling.

Kinetics

Perfect Agitation

The liquid or gaseous sample is perfectly agitated- all the analytes present in the sample have access to the fiber coating.

$$t_e = t_{95\%} = \frac{2(b-a)^2}{D_f}$$

Estimate the shortest equilibration time possible by substituting appropriate data for the diffusion coefficient of an analyte in the coating (D_f) and the fiber coating thickness (b-a)

Practical Agitation

Independent of the agitation level, fluid contacting a fiber's surface is always stationary, and as the distance from the fiber surface increases, the fluid movement gradually increases- until it corresponds to bulk flow in the sample. This static layer zone = the boundary layer and it's thickness is determined by the agitation conditions and viscosity of the fluid.

$$t_e = t_{95\%} = 3 \frac{\delta K_{fs}(b-a)^2}{D_s}$$

(b-a) is the fiber coating's thickness, D_s is the analyte's diffusion coefficient in the sample fluid, K_{fs} is the analyte's distribution constant, δ is the boundary layer thickness.

SPME Sampling

Three basic modes of SPME sampling:

Direct Extraction Mode:

- Coated fiber is inserted into sample and analytes are transported directly from the sample matrix to the extracting phase
- Rapid extraction facilitated by agitation
- For gaseous samples, convection is usually sufficient to facilitate rapid equilibration but for aqueous matrices more efficient agitation needed

Headspace Extraction Mode:

- Analytes extracted from the gas phase that is equilibrated with the sample
- Protects fiber from adverse effects caused by sample matrix
- Allows matrix modifications without affecting the fiber
- Sensitivity is the same as direct extraction, as long as sample and gaseous headspace volumes are the same
- Extraction kinetics are different than in direct extraction mode

Membrane-Protected Mode:

- Fiber is separated from the sample with a selective membrane, lets analytes through while blocking interferences
- Protects fiber from adverse effects caused by sample matrix
- Serves same purpose as headspace mode except that it can still analyze compounds having a low volatility
- Extraction process substantially slower than direct extraction

In-tube SPME (another type of sampling, usually used in the headspace mode)
Extraction phase remains in tubing during extraction- fiber retracted into needle or coating on inner tubing of needle. Therefore, the analyte concentration is measured at a well-defined place in space and time and long-term (integrated) sampling is possible, that accounts for analyte concentration changes with time and place to place variations in field analysis.

SPME: Experimental Variables

Coating Materials

Selectivity can be enhanced by choosing a coated fiber similar in chemical structure to the analyte

- Poly(dimethyl)siloxane- used for alkyl benzenes, PAH's, and volatile halogenated compounds
- Polyacrylate, or mixture of polyacrylate with Carbowax and/or polydivinylbenzene- used for alcohols and small polar compound

Increasing coating thickness, increases V_f and extracts a higher proportion of the analyte

Agitation Methods

As mentioned above, it changes the kinetics and therefore the equilibrium time. Sonication is the best method to reduce t_e.

Salting-out Effect

Addition of an inorganic salt to the aq. sample shifts the partition equilibrium so more analytes are extracted.

Effect of pH

Unless ion exchange coatings are used, SPME can extract only neutral (non-ionic) species. To ensure that at least 99% of acidic compound is in the neutral form, pH should be at least two units lower than pK_a of the analyte and the same goes for a basic compound, the pH should be at least two units higher than the pK_b of the analyte.

Sample Heating

Heating liquid samples gives faster diffusion rates of analytes (to coated surface)- therefore, reducing time needed for equilibrium, but at high temperatures less analyte is extracted. The best method to reduce equilibrium time and still extract sufficient analytes is to use an internally cooled fiber (via an inner capillary of liquid CO_2) while heating the solution.

Derivatization

This experimental variable is necessary for extracting and separating polar compounds. Derivatization can occur by: 1) adding appropriate reagents to matrix, followed by extraction; 2) doping fiber with the reagents, followed by extraction; 3) by extracting then exposing the fiber to derivatizing reagent; 4) by derivatizing within GC inlet.

Interfaces to Analytical Instruments

The GC is used most frequently with SPME because standard GC injections can be applied (without a special interface) as long as a narrow insert exists with an i.d. similar to the o.d of the SPME needle.

SPME/HPLC interface- addresses the need for analysis of nonvolatile and thermally labile analytes. Desorption loop placed in the position where the injection loop normally is. When the injection valve is at the load position, the fiber is introduced into the desorption chamber.

SPME/Optical interface- based on reflectometric interference spectrometry. If any of the extracted analytes strongly absorb the transmitted light, there is a loss in intensity which is detected with a simple optical sensor.

SPME/CE interface- facilitates direct insertion of fiber into inlet so analytes are desorbed directly in capillary, zero dead volume connection is accomplished.

SPME Advantages

- It is an equilibrium technique and is therefore, selective
- Time required for analyte to reach an equilibrium between the coated fiber and sample, relatively short
- Ideal for field sampling: large volume sampling, direct sampling, portable apparatus
- Solvent-less extraction and injection, eliminating solvent disposal
- Smooth liquid coating can be used, eliminating the problem of plugging
- By sampling from headspace, SPME can extract analytes from very complex matrices
- All analytes collected on the solid phase can be injected into GC for further analysis
- Method is fast, inexpensive, and easily automated
- Simple

SPME Disadvantages

- Often only a small fraction of the sample analytes are extracted by the coated fiber
- Quantification in SPME requires calibration
- Carryover resulting from incomplete desorption
- Fiber easily broken
- Limited number of polymeric coatings for SPME- lack of fibers that are sufficiently polar

Applications

Food and Pharmaceuticals:
Advantage in this area is that SPME can extract substances without opening the package. Furthermore, an insignificant amount is extracted and composition of the product does not change.

Environmental:
SPME can meet U.S. EPA method requirements with its low LOD's. These low detection limits reflect that all of the extracted analytes are introduced into the analytical instrument.

Clinical and Forensic:
The major advantage of SPME in this field is its portability: allows for better monitoring of patients during treatment or therapy and better preservation of crime scenes (evidence doesn't need to be taken back to the lab for analysis).